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_____ Date	_____ Signature

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of: KINGSMAN et al.	
Serial No.: 09/445,375	Group Art Unit: 1633
Filing Date: March 21, 2000	Examiner: Drabik, C.
Title: VECTOR	

**DECLARATION OF MILES CARROLL, Ph.D.**  
**SUBMITTED PURSUANT TO 37 C.F.R. § 1.132**

Commissioner for Patents  
Washington, D.C. 20231

Sir:

1. I am an inventor of the subject matter claimed in the U.S. patent application identified above.
2. Reference may be had to my *curriculum vitae*, attached hereto as Appendix A, for detailed information concerning my education and work experience. Briefly, I received my Bachelor of Science degree in Microbiology from University of Surrey and Ph.D. in Recombinant Vaccines following completion of studies at University of Manchester. I have 14 years of experience in molecular and cellular biology and have obtained numerous publications in peer reviewed journals in this field. I am currently VP Immunotherapy at Oxford BioMedica.
3. My patent application is premised on the important and unexpected finding that a vector comprising a polynucleotide encoding a tumor-binding protein which, for example, recognises a

trophoblast cell surface antigen such as 5T4 may be used to deliver a second polynucleotide of interest to the tumor.

4. The following data is provided demonstrating intra-tumoural delivery of genes which code for scFv proteins specific to 5T4 and expression therefrom.

#### **Example I Gene Transfer of scFv proteins *in vivo***

The aim of this study was to verify the expression of scFv following intratumour administration of a viral vector encoding scFv that has been tagged with myc and His polypeptides.

#### **Experimental Design**

An adenoviral vector expressing murine B7.1 fused to scFvmycHis (AdB7-scFv) was used to demonstrate the intra-tumoural delivery of genes that encode for scFv proteins specific to 5T4. A control adenoviral vector expressing the *lac z* gene (Adlac z) was also used. Both vectors used were of the human Ad5 serotype that lacks the genes encoding E1 and E3.

The coding sequence of B7-scFv used to make the adenoviral recombinant AdB7-scFv is the same as that described in Example 5 of the present patent application. Briefly the B7-scFv sequence was inserted into the *Hpa I* site of the transfer vector AdApt (Crucell, Netherlands) to produce AdAptB7scFv. AdAptB7scFv was cotransfected into the 911 derived helper cell line Per.C6 (Crucell) with AdEasy genome plasmid (Qiagen) to produce the adenoviral recombinant.

Cells of the murine cancer cell line CT26 expressing h5T4 (CT26-h5T4) were washed twice in PBS and  $5 \times 10^5$  cells were injected sc into both flanks of a female Balb/c mouse. Each tumour was established to an average diameter of 5mm and received 3 daily injections of  $4 \times 10^8$  pfu Adenovirus in 50 $\mu$ l. 48 hours after the final injection, tumours were excised and snap frozen and 100 8 $\mu$ m sections were cut prior to staining for murine B7.1 or c-myc proteins.

## Results

### Figure A: Immunostain of CT26-h5T4 tumours injected with Adlac z

There was no positive staining for c-myc or B7.1 in sections adjacent to those that were positive for lac z. This confirms the specificity of staining by the anti-bodies used and also verifies that B7.1 positive staining seen in Figure B is due to gene transfer of the scFv fusion protein and not the presence of adenovirus.

### Figures B and C: Immunostain of CT26-h5T4 tumours injected with AdB7-scFv

Gene transfer of B7-scFv was verified on adjacent sections that stained positive for B7.1 as well as for the c-myc tag. Specificity of staining was verified by the absence of positive cells in those sections that were incubated in the secondary antibody alone.

Results of the immunostaining experiments in figure C are summarised in Table 1:

<b>OBM Number</b>	<b>Treatment</b>	<b>X-gal Staining Results</b>	<b>C-myc staining results</b>	<b>B7.1 staining results</b>
02.0104	Untreated 5T4 Tumour	Negative	Negative	Negative
02.0105	5T4 AdB7LScFv	Negative	Slides 1-54, sections ranging from 1-5% positive	Slides 1-54, sections ranging from 1-20% positive
02.0107	Neo Ad.Lac Z	Slides 1-54, sections ranging from 5-30% positive	Negative	Negative

In summary, as shown in Table 1 and Figures B and C, the B7.1 scFv was expressed in the tumour following intratumoural injection.

**Example 2 Gene Transfer of B7 scFv proteins in sera of Balb/c mice.**

In this study, the AdB7-scFv vector construct as described above in Example 1 was injected iv into Balb/c mice. Control mice were injected with AdlacZ construct prepared in the same manner.

Briefly,  $4 \times 10^8$  pfu of virus diluted in PBS was injected iv in a final volume of 100 $\mu$ l. At 2 days, 4 days and 7 days, serum samples from individual mice were diluted 1:32, 1:64 and 1:128. B7-scFv specific to 5T4 was measured in the sera using a capture ELISA where 50 $\mu$ l/well of diluted sera was added to plates previously coated with 100 $\mu$ l/well of 1 $\mu$ g/ml human 5T4 overnight at 4°C. Plates were blocked with PBS containing 2%FBS and 0.1% Tween (blocking buffer) for 2 hours at room temperature. B7-scFv was detected using 100 $\mu$ l/well of an antibody specific to murine B7.1 (rat anti-CD80, Pharmingen) diluted to 0.5 $\mu$ g/ml in blocking buffer. Following incubation for 1 hour at room temperature, ELISA plates were washed 6 times with 200 $\mu$ l/well PBS containing 0.1% Tween. This was followed by 100 $\mu$ l/well of antibody conjugated to HRP and specific to rat (DAKO labs) diluted to 1.3 $\mu$ g/ml in blocking buffer. After 1 hour at room temperature plates were washed as described above and peroxidase activity determined using 100 $\mu$ l/well of substrate TMB (Biosource International). The enzyme reaction was stopped using 50 $\mu$ l/well of 2M HCl. Values obtained from sera diluted 1:32 were used to determine the final concentration of B7.1scFv in the serum of each mouse injected by subtracting the values obtained from mice injected with AdlacZ from those obtained from mice injected with AdB7-scFv. This is summarised in Table 2.

As shown in Figure D and Table 2, B7.1 was detected in the sera of the injected mice at each of the time points post-injection, demonstrating gene transfer of the B7-scFV specific to 5T4.

Table 2: B7.1scFv (ng/ml) detected in sera of Balb/c mice following injection of AdB7-scFv iv

Adenoviral construct injected	Time post injection (day)	OD at 405 nm	B7.1scFv (ng/ml)	Normalised B7.1scFv (ng/ml)
AdLac z	2	0.607	47.12	0
AdB7.1scFv	2	0.991	77.11	30.0
AdLac z	4	0.603	45.53	0
AdB7.1scFv	4	1.067	80.65	35.1
AdLac z	7	0.521	52.402	0
AdB7.1scFv	7	0.628	63.20	10.8

**Example 3 The scFv-Hy1 fusion protein is able to direct ADCC lysis of 5T4 positive human tumour cells.**

A scFv-Hy1 fusion protein was constructed as described in Example 12 of the present patent application and produced as described in Example 15.

A chromium release assay demonstrates that the scFv-Hy1 fusion protein can direct cytotoxicity against cells expressing the 5T4 antigen at the cell surface.

A9-5T4 and A9-neo cells were labelled with  $^{51}\text{Cr}$  and then incubated with either scFv alone or scFv-Hy1. Increasing amounts of peripheral blood lymphocytes were used as the effector cells and lysis allowed to proceed for 4hrs. Chromium released into the supernatant was measured by scintillation counting to assess the extent of lysis achieved.

As shown in Figure E, there is increasing lysis of the cells with increasing protein concentration and also with an increasing effector to target ratio. This is clearly 5T4 specific when the A9-5T4 cells are compared with the negative control A9-neo. In addition the results using the scFv alone clearly indicate that the lysis is directed by the Fc portion of the fusion protein.

**Example 4 Genetic delivery of 5T4scFv- Hy1**

The murine colon cancer cell line, CT26, was engineered to express either human 5T4 (and neomycin resistance), or neomycin resistance alone. These were transduced with an MLV based fusion construct ,MLV-LscFvHy1 (produced as described in Example 15, using the triple plasmid HIT system with pLXSN/ScFv-IgG) and analysed by FACS.

Using a goat anti-HIgG-FITC antibody, HIgG is detected at the surface of the CT26-5T4 cells but not the CT26-neo cells (Figure F). This demonstrates that genetic delivery of the fusion protein construct leads to secretion of the protein from the cell and binding back to the cell surface if 5T4 antigen is present in the membrane.

### **Summary**

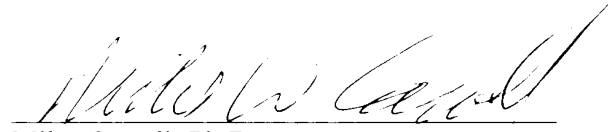
The above described results clearly support that:

- (i) Vectors comprising a polynucleotide encoding a tumour interacting protein may be used to deliver a second nucleotide to the tumour cell; and
- (ii) The delivered second polynucleotides of interest may be expressed leading to secretion of a protein of interest which can then bind back onto the cells and lead to, for example lysis of the tumour cells.

Accordingly, all claims are enabled by the present application as filed.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 16<sup>th</sup> April 2002

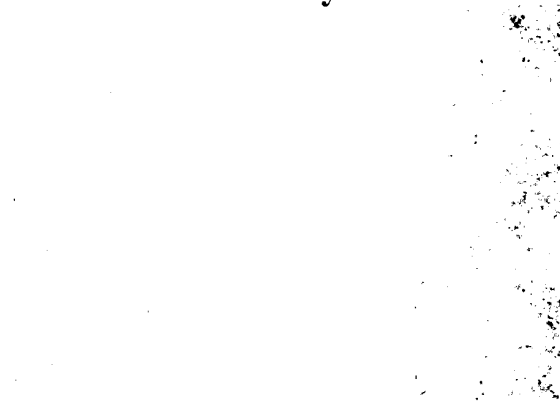
  
Miles Carroll, Ph.D.

**Figure A**

**anti B7.1**



**anti c-myc**



**X-GAL**

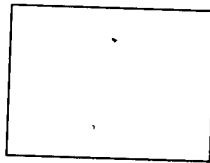




**Figure B(1)**

**anti B7.1**

**X100 mag**

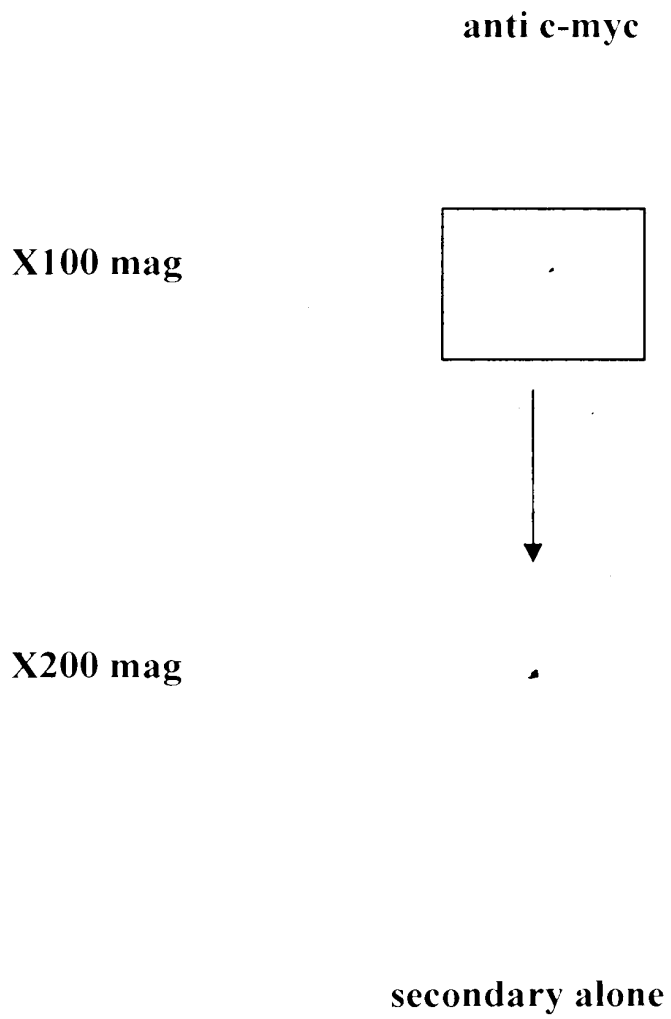


**X200 mag**

?

**secondary alone**

**Figure B(2)**



**Figure C- B7.1 Immunostaining of B7.1 in tumours following intratumoural injection of AdB7-scFv vectors.**



Positive and negative control cell pellets shown at x100 magnification



02.0105 5T4 tumour injected with Ad.B7LScFv (shown at x40 and x200 magnification)

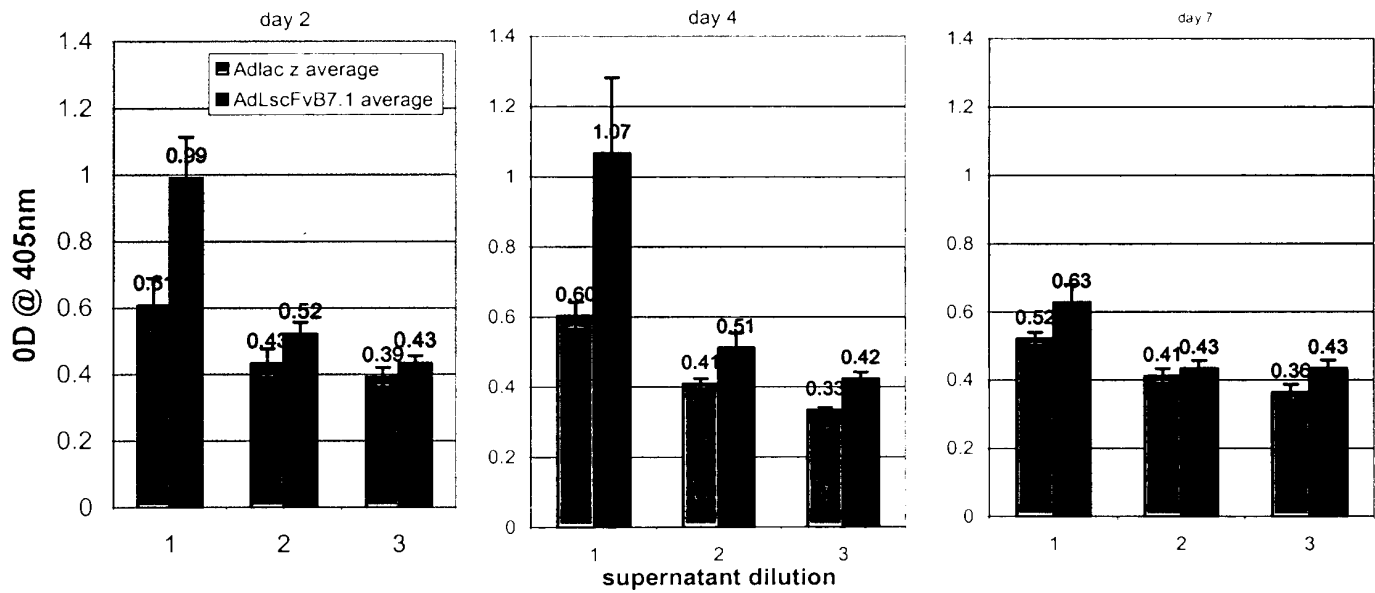
**Figure D**

**Detection of B7.1 in the sera of mice injected with AdB7LscFv**

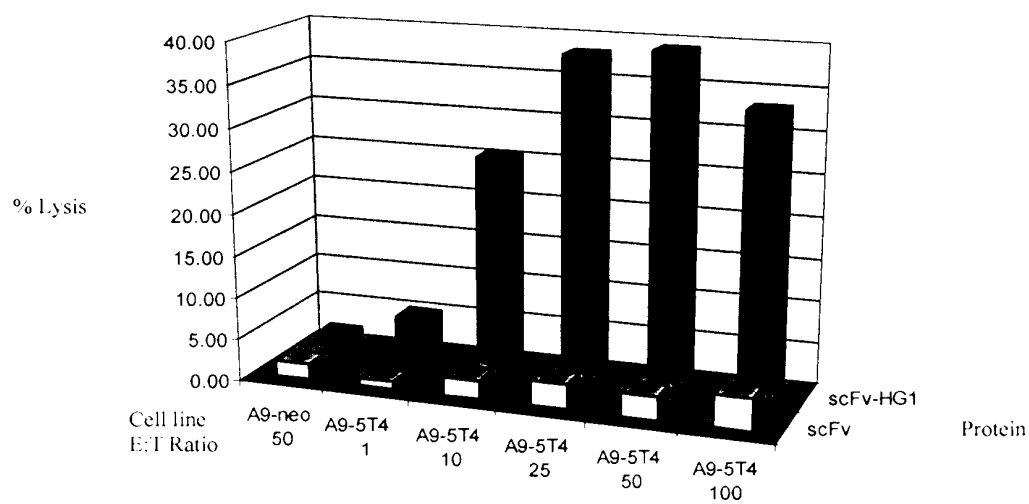
**Average of 3 mice (Adlac z) or of 5 mice (AdB7scFv)**

dilution1=1/32, dilution 2= 1/64, dilution 3= 1/128

SEM for each group are shown

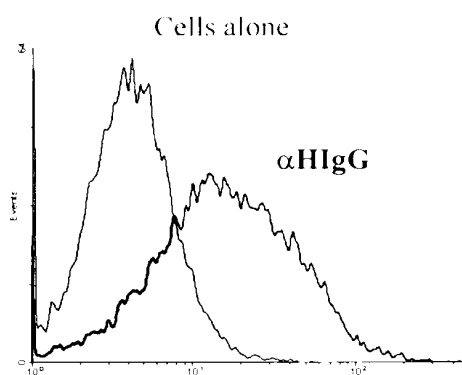


**Figure E Antibody Dependent Cell Cytolysis of 5T4 expressing cells**

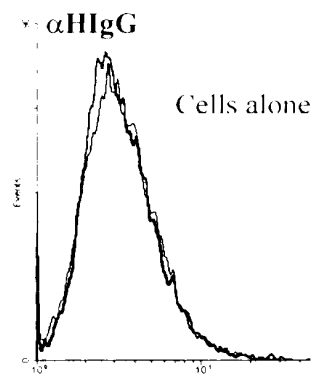


**Figure F FACS analysis of cells transduced with the MLV-scFv-H $\gamma$ 1 construct**

**A: CT26-5T4**



**B: CT26-neo**



CT26-5T4 (**A**) or CT26-neo cells (**B**) were transduced with an MLV based construct expressing scFv-H $\gamma$ 1. Goat anti-HlgG-FITC was used to detect endogenously produced fusion protein that was secreted from the cells and had subsequently bound to the cell surface.

## APPENDIX A

### Curriculum Vitae: Miles William Carroll PhD

**Home Address:** 59 Mably Grove  
Wantage  
Oxon  
OX12 9XW

**Work:** Oxford Biomedica  
Medawar Centre  
Oxford Science Park  
Oxford  
OX4 4GA

**Tel:** 01235 760901

01865 783000

**Fax:** 01865 783001

**email:** m.carroll@oxfordbiomedica.co.uk

**Personal:** **Date of Birth:** 10.10.65.

**Nationality:** British

#### Education:

**1984-1988: University of Surrey:**

B.Sc. Hons. Upper Second Class in Microbiology.

**Oct. 1989-Oct 1992: University of Manchester:**

PhD student at the Paterson Institute for Cancer Research (Manchester University, Dept. Oncology) under the supervision of Dr Michael Mackett. PhD Thesis: Expression, analysis and immunogenicity of human immunodeficiency virus type 1 envelope glycoprotein in vaccinia virus.

## **Employment:**

**August 1997-Present.** Vice President, Tumour Immunotherapy, Oxford Biomedica, Oxford Science Park, Oxford, OX4 4GA, UK.

**July 1993-July 1997:** Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda MD 20892, USA.

Postdoctoral Research Fellow (International Fogarty Fellowship) working with Dr **Bernard Moss** at the National Institutes of Health within the NIAID, USA. Development of recombinant poxvirus vectors to improve immune induction properties. SIV vaccine development. Poxvirus host range. Development of poxviruses as vectors for cancer immunotherapy.

**August 1988-September 1989:** Research Assistant, Dept. of Molecular Biology, Paterson Institute (Manchester University, Dept. Oncology). Development of recombinant vaccinia virus transfer vectors.

**September 1986-September 1987:** Medical Laboratory Scientific Officer, Dept. of Medical Microbiology, St. Mary's Hospital Medical School, London.

**September 1982-September 1984:** Intervet Laboratories International, Cambridgeshire, UK. Research Assistant.



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22. **MW Carroll** & NP Restifo 2000 Poxviruses as vectors for cancer immunotherapy In *Cancer Vaccines and Immunotherapy* pp 47-61 Ed. PL Stern, PCL Beverly & **MW Carroll** Cambridge University Press, Cambridge UK
21. PCL Beverly, PL Stern & **MW Carroll** 2000 Immunity and Cancer pp 1-18 *Cancer Vaccines and Immunotherapy* Ed. PL Stern, PCL Beverly & **MW Carroll** Cambridge University Press, Cambridge UK
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**MW Carroll** & KM Myers 2000 "Polypeptide" WO 00/29428.

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## References

Bernard Moss MD PhD  
Lab of Viral Diseases  
NIAID  
National Institutes of Health  
Bethesda  
MD 20892  
Bmoss@nih.gov

Mike Mackett PhD  
Dept. Molecular Biology  
Paterson Institute  
Withington  
Manchester  
M20 9BX  
mlbmm@picr.cr.man.ac.uk  
0161 446 3157

John Williamson PhD  
Senior Lecturer  
Virology Dept.  
St Mary's Hospital Medical School  
Paddington  
London  
W2  
jd.williamson@ic.ac.uk